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A peptide vaccine administered transcutaneously together with cholera toxin elicits potent neutralising anti-FMDV antibody responses

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Abstract

In this study a synthetic peptide representing residues 141–159 from the GH loop of VP1 protein of foot-and-mouth disease virus was tested for its capacity to elicit virus neutralising antibodies in mice after transcutaneous immunisation. Topical application of the peptide conjugated to bovine serum albumin together with cholera toxin as an adjuvant elicited anti-peptide antibody responses with strong virus neutralising activity. The combination of cholera toxin with an immunostimulatory CpG motif resulted in the induction of IgG1 and IgG2a anti-peptide antibodies with significantly enhanced virus neutralising activity. To shed more light on the mechanisms of cholera toxin adjuvanticity we demonstrated its binding to keratinocytes via GM_1-gangliosides. This was followed by an increase of the intracellular cAMP and the rapid diffusion of cholera toxin throughout the epidermis.

These findings demonstrate that peptide-based vaccines when combined with the appropriate adjuvant(s) can elicit potent virus neutralising antibody responses after transcutaneous immunisation. However, experiments in target species will be required to confirm the potential of this simple vaccination procedure in livestock.

Keywords: Transcutaneous immunisation; Peptide vaccines; FMDV; Cholera toxin; Adjuvants

1. Introduction

Foot-and-mouth disease virus (FMDV) causes a highly contagious disease of farm animals that almost invariably leads to considerable losses of productivity. Control in countries that are normally free from the

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disease is achieved by ''slaughtering on suspicion'', whereas vaccination is more commonly used in endemic areas. Current FMDV vaccines are prepared by chemically inactivating virus grown in tissue culture. This procedure poses the risk of virus escape from production plants (Brown, 1991) so alternative methods have been investigated during the past two decades. Among these is the use of peptides corresponding to the highly immunogenic GH loop sequence present on the VP1 capsid protein. Several groups have shown that this sequence elicits high levels of neutralising antibodies that provide protection upon challenge with autologous virus in various animal species (Pfaff et al., 1982; Bittle et al., 1982; Brown, 1992; Nargi et al., 1999; Fischer et al., 2003). In most of these experiments the peptide-based vaccine was administered by a subcutaneous or intramuscular injection. However, vaccination using needles and syringes can lead to transmission of blood-borne infections, formation of abscesses at the site of inoculation, and pose the risk of adverse reactions and reduction of meat and hide-quality. These potential problems have prompted us to examine a novel non-invasive vaccination strategy, coined the term transcutaneous immunisation (Glenn et al., 1998a). This procedure is based on the application of antigen onto the bare skin together with an ADP-ribosylating exotoxin as an adjuvant (Glenn et al., 1998a; Scharton-Kersten et al., 2000; Beignon et al., 2001). After its application the vaccine antigen diffuses through the epidermal layers to the basal layer, where Langerhans cells (LCs) reside. LCs are highly efficient at taking up antigen locally, then migrating to regional lymph nodes and activating naïve T cells. In several studies transcutaneous immunisation has been shown to induce protective immune responses in animal models (Glenn et al., 1998b; Beignon et al., 2001; Godefroy et al., 2003; Tierney et al., 2003). More recently, the effectiveness of this immunisation procedure was demonstrated in humans (Glenn et al., 2000; Guerena-Burgueno et al., 2002). For animal vaccination, transcutaneous immunisation has the potential to reduce carcass damage, simplify vaccine delivery, and improve animal welfare.

In this study, we sought to evaluate the immunogenicity of a candidate FMDV synthetic peptide vaccine after topical application together with cholera toxin (CT) for its ability to elicit virus neutralising antibody responses in mice. Moreover, we assessed by flow cytometry the capacity of CT to bind to keratinocytes and studied its distribution throughout the epidermis by immunohistochemistry.

2. Materials and methods

2.1. Peptide synthesis and conjugation

Peptide 141–159 from the VP1 protein of serotype A12 (Cys-^{141}GSGVRGDFGSLAPRVARQL^{159}) was prepared by solid phase synthesis using Fmoc chemistry (Briand et al., 1997). Following cleavage, the peptide was purified by preparative high performance liquid chromatography (HPLC) and characterised by HPLC and mass spectroscopy.

Peptide 141–159 was conjugated to amino-groups present on the carrier proteins bovine serum albumin (BSA) and ovalbumin (OVA) via the thiol (SH) group of the additional cysteine residue using *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester according to the manufacturer's instructions (Pierce, Rockford, IL). The same coupling method as described above was used for the conjugation of a control peptide from influenza virus nucleoprotein (NP: 55–69) to OVA.

2.2. Animals

Female BALB/c mice, 6–8 weeks old at the start of the experiments were purchased from Harlan (Gannat, France) and maintained in the animal facility of the Institut de Biologie Moléculaire and Cellulaire, Strasbourg, France.

2.3. Immunisation procedure

Prior to immunisation, a small surface area of the abdomen was shaved with a razor and hydrated for 5 min. After blotting the skin with a dry tissue the antigen solution (30 μl) was applied onto the bare skin of each mouse (6 mice per group) as a mixture of: (a) 100 μg unconjugated 141–159 peptide mixed with 100 μg CT (Sigma–Aldrich, St. Louis, MO); (b) 100 μg 141–159 peptide conjugated to BSA (total protein) together with 100 μg CT; (c) 100 μg 141–159 peptide conjugated to BSA together with 100 μg CT

and 100 μg of synthetic phosphorothioate-stabilised oligonucleotide containing the immunostimulatory CpG motif 1018 (ODN CpG 1018) (5′-TGA CTG TGA ACG TTC GAG ATG A-3′) (Eurogenetec, Seraing, Belgium); (d) 100 μg 141–159 peptide conjugated to BSA plus 100 μg CT and 100 μg ODN non-CpG 1745 (5′-TCC AAT GAG CTT CCT GAG TCT-3′) (Eurogenetec). In all cases, booster applications with the same dose and formulation were given on days 21, 42, and 63. During the immunisation procedure, mice were given sufficient anaesthetic to immobilise them for approximately 1 h (to limit grooming activity and to allow for antigen absorption) by injecting intramuscularly 150 μl solution of 1 ml ketamine [imalgene 1000 (15%), Merial, Lyon, France] + 0.6 ml xylasine [2% rompuin (9%), Bayer AG, Leverkusen, Germany] + 5 ml sterile phosphate buffered saline (PBS). One hour after skin exposure to the antigen, the site of topical application was thoroughly washed with water. It is important to note that there were no any apparent signs of skin inflammation during and after immunization.

2.4. ELISA for antibody responses

Anti-peptide antibodies in mouse sera were determined using an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtitre plates (Falcon, Oxnard, CA, USA) were coated overnight with 5 μg/ml of peptide 141–159 conjugated to OVA (as described above) in 0.05 M carbonate/bicarbonate buffer, pH 9.6 at 37 °C. The plates were blocked with 1% OVA in PBS containing 0.05% Tween 20 (PBS-T) at 37 °C, for 2 h. Following washing with PBS-T, serial two-fold dilutions of serum in PBS-T containing 0.25% OVA were made across the plate (final volume 50 μl) and plates were incubated at 37 °C for 1 h. After washing with PBS-T, 50 μl of 1/20 000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson Immuno-Research Laboratories Inc., West Grove, PA, USA) or 1/10 000 dilution of Fc-specific anti-IgG1 or IgG2a (Nordic Immunology, Tilburg, The Netherlands) were added to each well and the plates incubated at 37 °C for 1 h. Unbound conjugate was removed by washing with PBS-T and the enzymatic activity was determined by adding 150 μl/well of substrate solution [10% citric phosphate buffer pH 5 + 0.04% H_2O_2 + 90% of a solution containing 72 ml dimethylsulphoxide + 18 ml glycerol + 300 mg 3,3′,5,5′-tetramethylbenzidine (TMB)] for 15 min at 37 °C. The reaction was stopped by adding 25 μl of 0.25 M HCl, and the absorbance was measured at 450 nm. Data are expressed as antibody titres corresponding to the reciprocal dilution giving an OD $\geq$ 0.2. Antibody titres to the control peptide NP:55–69 conjugated to ovalbumin (55–69-OVA) were subtracted from titres measured against the plate-bound antigen 141–159-OVA to offset the contribution made by the linker used to conjugate the peptide to BSA.

2.5. Neutralisation assay

Neutralisation of FMDV in vitro was performed as described previously (Briand et al., 1997). Briefly, equal volumes of 10-fold dilutions of virus and 1/50 serum were preincubated at 37 °C for 20 min before adding the mixtures to monolayers of LF-BK cells in 96-well plates. After a three-day incubation in a 5% CO_2 atmosphere the cells were stained with crystal violet-Histochoice. Results were expressed as neutralisation indices (a neutralising index of 2 means that a 1/100 dilution of an anti-serum reduces the titre of the homologous virus by 2 $\log_{10}$ units).

2.6. Cell culture and treatment of HaCaT cells

HaCaT keratinocyte cells (kindly provided by Dr. N. Fusening, German Cancer Research Center, Heidelberg, Germany) were grown in 75 cm^2 culture flasks (Costar, Cambridge, MA, USA) and maintained in Dulbecco's modified Eagles's medium (DMEM; BIO MEDIA, Boussens, France) supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml gentamycin and 25 mM HEPES (BIO MEDIA) in a humidified atmosphere containing 5% CO_2 at 37 °C. At confluence, the supernatant and floating cells were removed and adherent cells were detached using a mixture of 0.025% EDTA/0.05% trypsin (BIO MEDIA).

HaCaT cells (5×10^5) were incubated in glass tubes with 0.5 μg of FITC-conjugated to cholera toxin B subunit (CTB-FITC, purchased from Sigma) in PBS/2% FCS at 4 °C for 30 min. After incubation the excess of CTB-FITC was removed by washing the cells with PBS/2% FCS and binding was monitored by

flow cytometry using a FACScalibur (Becton Dickinson, Mountain View, CA, USA) and CELLQuest™ software (Becton Dickinson). To inhibit the binding of CTB-FITC keratinocytes were incubated with various mixtures of CTB-FITC and GM_1 type III gangliosides (Sigma) preincubated at 4 °C for 24 h before addition to the cell culture.

2.7. Measurement of cyclic AMP

HaCaT cells were seeded into 24-well tissue culture plates. When they reached confluence cells were cultured in serum free DMEM for 3 h. Then they were incubated with 1 ml of HBSS medium (BIO MEDIA) buffered with 10 mM of HEPES containing 100 μM of 3-isobutyl-1-methylxanthine, an inhibitor of the phosphodiesterase to block camp hydrolysis (IBMX, Sigma). After 15 min, various concentrations of CT were added to the cells and plates were incubated at 37 °C for 30 min. The reaction was stopped by removing the stimulatory solution and adding boiling water. Samples were frozen at − 20 °C until assayed. Levels of cAMP were determined using a competitive assay (Biotrack™ cAMP, Amersham Pharmacia Biotech, Orsay, Paris) according to the manufactures' instructions. The protein concentration in each sample was measured with a BCA-200 protein assay kit (Pierce, Rockford, IL, USA).

2.8. Skin collection and preparation

Skin biopsies (taken from the site of antigen deposition) were collected at various time intervals after topical application of 100 μg CT. Skin was excised and washed thoroughly with chilled PBS. Thereafter, it was placed overnight in a solution of 4% saline-formaldehyde at room temperature. The fixed tissues were dehydrated and embedded in paraffin according to a standard procedure.

2.9. Immunohistochemistry

Skin was sectioned at 4 μm and sealed to Superfrost Plus (CML, France) slides. Then the slides were allowed to dry overnight at 58 °C. After removal of the paraffin and rehydration, slides were incubated in Tris-buffered saline (TBS) for 10 min, transferred to the blocking buffer (TBS, 5% low fat skimmed milk, 0.1% Tween 20) for 1 h at room temperature, and then incubated with 1/50 dilution of mouse anti-CT polyclonal serum at room temperature, overnight. Following washing with TBS, the slides were incubated with a biotinylated goat anti-mouse antibody conjugated to horseradish peroxidase (ABC elit, AbCys, France) at room temperature for one hour, then washed extensively with TBS. The CT was then detected by incubating the slides with diaminobenzidine (DAB) substrate solution until the development of adequate colour had occurred.

3. Results

3.1. Antibody responses after transcutaneous immunisation with peptide 141–159 conjugate

In preliminary experiments, the immunogenicity of unconjugated 141–159 peptide was tested after transcutaneous immunisation with CT. No anti-peptide antibody responses were detected (data not shown). However, conjugation of the 141–159 peptide to BSA rendered it immunogenic. Groups of mice immunised transcutaneously with the conjugated peptide + CT or mixtures of CT + ODN CpG 1018 or CT + ODN non-CpG 1745 as adjuvants, seroconverted (Fig. 1). Titres of anti-peptide antibodies increased after the second boost, whereas the third boost increased further the antibody responses of mice immunised with the conjugated peptide + CT or CT + ODN non-CpG 1745. The predominant antibody

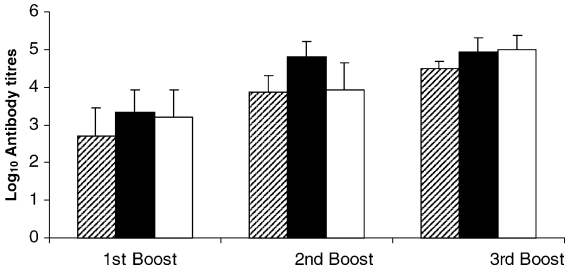


Fig. 1. Anti-peptide antibody responses after transcutaneous immunisation of groups of six BALB/c mice with peptide 141–159 conjugated to BSA together with CT (▨) or CT + ODN CpG 1018 (■) or CT + ODN non-CpG 1745 (□). Serum samples drawn one week after each booster administration were tested by ELISA.

subclass (after the third boost) in the presence of CT was IgG1 (ratio of IgG1/IgG2a = 22.5) whereas the combination of CT and ODN CpG 1018 shifted the subclass profile towards a more balanced response (ratio of IgG1/IgG2a = 5).

Virus neutralising antibody titres were found in the serum of groups of mice immunised with the conjugated 141–159 peptide + CT or the mixture of CT + ODN CpG 1018 after the first boost (Fig. 2). Following the second boost, the neutralising indices were significantly increased ($P = 0.01$, according to Student's *t*-test) in the group of mice receiving the CT and ODN CpG 1018 mixture as an adjuvant. Furthermore, these responses were significantly higher than those elicited in the presence of CT ($P = 0.01$) or CT and ODN non-CpG 1745 ($P = 0.02$) (Fig. 2). Surprisingly, neutralising titres were very low in the group of mice immunised with the conjugated 141–159 peptide + CT + ODN non-CpG 1745 (Fig. 2) despite the presence of high anti-peptide antibody titres (Fig. 1).

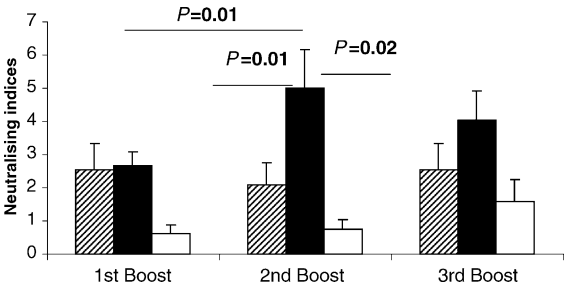


Fig. 2. Neutralisation indices of anti-peptide antibody responses elicited after transcutaneous immunisation of groups of six BALB/c mice with BSA-conjugated 141–159 peptide together with CT (▨) or CT + ODN CpG 1018 (■) or CT + ODN non-CpG 1745 (□). Serum samples drawn one week after each booster administration were tested for neutralising activity.

3.2. Binding of CTB to keratinocytes

One of the factors that influence the immunogenicity and adjuvanticity of CT is its capacity to bind to GM_1-ganglioside receptors on cell membranes via

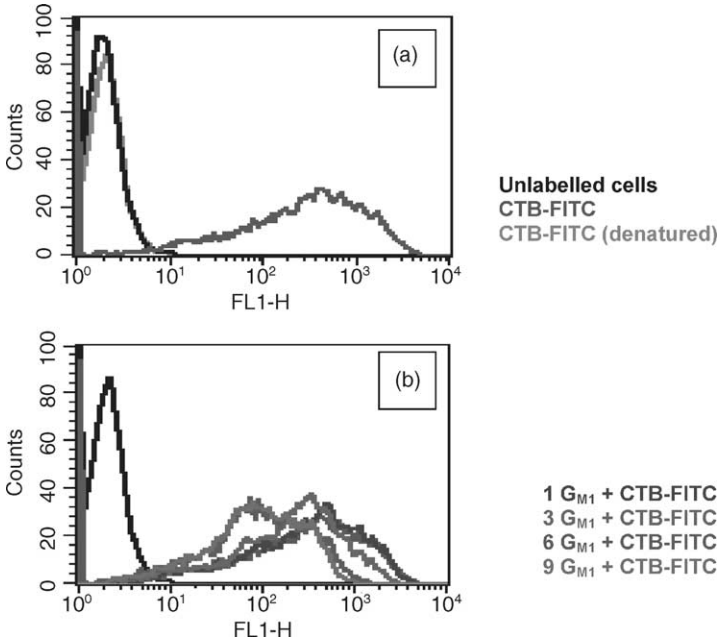
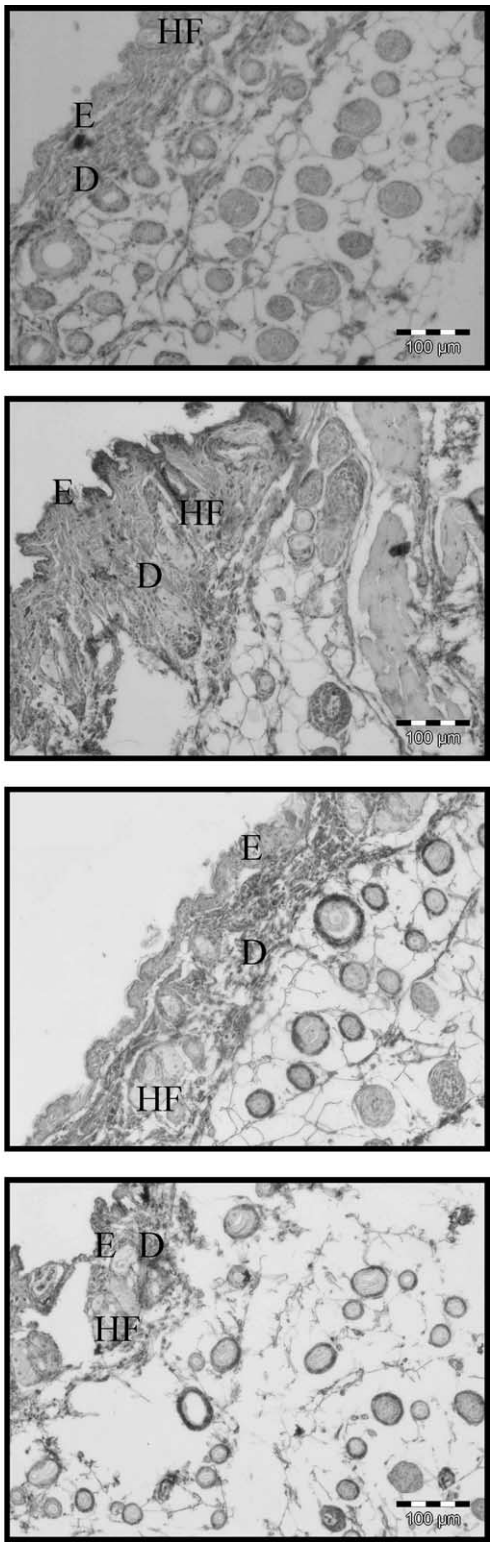


Fig. 3. Binding of CTB to HaCaT keratinocytes. Cells were incubated with 0.5 μg of CTB-FITC (red) or denatured after boiling (blue) (a) or with CTB-FITC preincubated with various concentrations of GM_1 type III gangliosides (b); CTB-FITC/GM_1 (w/w ratio); 1 μg:1 μg (green, the mean fluorescent intensity was 263), 1 μg:3 μg (dark blue, the mean fluorescent intensity was 184), 1 μg:6 μg (violet, the mean fluorescent intensity was 92), 1 μg:9 μg (orange, the mean fluorescent intensity was 84). Cells were washed to remove the excess of unbound CTB-FITC and then analysed by flow cytometry. The autofluorescence of unlabelled cells or cells incubated with GM_1 type III ganglioside is represented in black. The presented results are representative of three independent experiments.



CTB (Scharton-Kersten et al., 2000; Beignon et al., 2001). In this study we analysed the binding of CTB to HaCaT keratinocytes by flow cytometry. Fig. 3a shows that CTB-FITC binds to keratinocytes and this binding required the native conformation of the molecule. When CTB was denatured no binding was observed. The binding of CTB was reduced after preincubation of CTB-FITC with various excesses of GM_1 type III ganglioside (Fig. 3b). The binding of CT to HaCaT keratinocytes was followed by activation of adenyl cyclase, an enzyme responsible for the observed increase of cAMP that was dose-dependent (12, 13, 34 and 38 fmol/ml cAMP in the presence of 0, 0.05, 0.1, and 10 μg/ml of CT, respectively).

3.3. Skin distribution of CT after transcutaneous immunisation

A rapid diffusion of CT throughout the epidermis and dermis was observed five minutes after its application onto the pre-hydrated bare skin of mice as judged by the homogeneous staining of the tissue (Fig. 4). The distribution of CT around the hair follicles was particularly notable. Skin biopsies taken 30 min and 1 h after topical application revealed a much more dispersed pattern of CT staining in the epidermis.

4. Discussion

Synthetic peptide immunogens have been shown to be promising candidate vaccines against FMDV (Bittle et al., 1982; Nargi et al., 1999; Wang et al., 2002; Fischer et al., 2003). In the present study we examined the immunogenicity of serotype A12 141–159 peptide vaccine conjugated to BSA after transcutaneous co-immunisation with CT or with a mixture of CT and an ODN CpG. Strong serum anti-peptide antibody responses were induced that had virus neutralising activity. Although the biological

Fig. 4. Distribution of CT in the skin after its topical application. This was assessed by using a mouse anti-CT polyclonal serum: (a) normal skin; (b) distribution of CT, 5 min after its topical application; (c) distribution of CT, 30 min after its topical application; (d) distribution of CT, 1 h after its topical application. Prior to topical application of CT, the skin was shaved and hydrated for 5 min. D = dermis, E = epidermis, HF = hair follicles.

relevance of these antibodies has not been tested in vivo, it is known that there is a good correlation between serum neutralising antibody titres and protection (Wang et al., 2002). However, the neutralising capacity of antibodies is not the only parameter of the protective responses to FMDV. Antibody avidity and isotype responses have also been suggested to be important correlates for biological effectiveness (Mulcahy et al., 1990; Steward et al., 1991).

The exact mechanisms of the adjuvanticity of CT are still not clear. In this study CT was shown to bind to epidermal keratinocytes via the GM_1-gangliosides, which is a critical first step for its immunogenicity (Beignon et al., 2001). Moreover, when CT was applied onto pre-hydrated bare skin it rapidly diffused throughout the epidermis. Following the binding of CT to keratinocytes levels of cAMP were increased. This may constitute a signal to keratinocytes that stimulate the secretion of proinflammatory cytokines like IL-1 and TNF-α, which in turn act on LCs to trigger their maturation and migration to regional lymph nodes (Wang et al., 1999). CT has also been shown to act directly on LCs promoting their activation and migration to regional lymph nodes (Guebre-Xabier et al., 2003) or mucosal sites (Enioutina et al., 2000).

Combination of CT with the immunostimulatory ODN CpG 1018 significantly enhanced the anti-virus neutralisation titres and induced both IgG1 and IgG2a in the serum, which is indicative of a mixed Th1–Th2 response. This immune enhancement suggests that CT and CpG act synergistically. This finding is in agreement with observations demonstrating the synergistic adjuvant activity of CT/ODN CpG mixtures after transcutaneous co-immunisation with peptide antigens (Beignon et al., 2002; Partidos et al., 2004). Surprisingly, co-immunisation with the non-immunostimulatory ODN CpG 1745 resulted in the induction of very low anti-virus neutralising titres despite the presence of high anti-peptide antibody responses. The immunostimulatory effect of the non-CpG motif (as evident by the increase of anti-peptide antibody titres) could be attributed to the presence of skin immune defence mechanisms that are less specific but more vigorous than those found at other anatomical sites to combat ubiquitous pathogens encountered at this surface. A similar effect was also observed in a recent study where the combined adjuvanticity of CT and CpG was studied after transcutaneous immunisation with the outer membrane protein of *Chlamydia muridarum* (Berry et al., 2004).

In conclusion, the present study has demonstrated that transcutaneous immunisation with peptide antigens together with the appropriate combination of adjuvants can elicit potent FMDV neutralising antibody responses. The validity of this vaccination procedure could be easily tested in livestock since species-specific immunostimulatory CpG sequences have already been identified (Mutwiri et al., 2003). In addition, high doses of CT when delivered via the skin do not exert any toxic effects that are normally observed after its mucosal delivery. However, we should be cautious in extrapolating the findings of studies performed in different animal species to the target species because of differences in anatomy and physiology of the skin between species. Despite the challenges, the potential returns of transcutaneous delivery of vaccines makes it a goal worthy to pursue. Furthermore, novel technological advances in transdermal delivery that partially disrupt or porate the skin barrier promise to make skin vaccination a realistic option for future vaccination (Partidos, 2003).

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